ANTISENSE OLIGONUCLEOTIDES AND USES THEREOF IN IMPROVING CANCER TREATMENT STRATEGIES

FIELD OF THE INVENTION

The present invention pertains to the field of cancer therapies and in particular to antisense oligonucleotides for use improving cancer therapies.

BACKGROUND

The use of antisense oligodeoxynucleotides (ODNs) as therapeutic molecules is known. Several antisense ODNs targeting a variety of molecules have been shown to have antiproliferative effects against neoplastic cells *in vitro* and *in vivo* (Gewirtz, 2000, *J.Clin.Oncol.* **18**:1809-1811), and several have demonstrated anti-tumour activity and limited toxicity in Phase I clinical trials (Smith and Wickstrom, 2000, *Methods Enzymol.* **314**:537-580).

There are a number of proteins that have been implicated in cancer and, as a result, have been targeted by cancer therapies using standard chemotherapeutics. An example is thymidylate synthase (TS), which is an essential enzyme in *de novo* production of thymidylate (Carreras and Santi, 1995, *Annu.Rev.Biochem.* **64**:721-762). Due to the crucial role of TS in DNA synthesis and cell proliferation, it has been an important target for cancer chemotherapy for many years (Danenberg, 1977, *Biochim.Biophys.Acta* **473**:73-92; Danenberg *et al.*, 1999, *Semin.Oncol.* **26**:621-631).

Chemotherapeutics that inhibit TS, such as 5-fluorouracil (5-FU) and its variants, have become integral drugs in standard treatments for colorectal cancer (Papamichael, 1999, *Oncologist.* **4**:478-487). Raltitrexed (Tomudex®) and pemetrexed (Alimta®) are other TS inhibiting chemotherapeutics with a potential role in a range of cancers including mesothelioma. Although reasonably successful in clinical use, these drugs suffer from problems of dose-limiting toxicity and outgrowth of resistant cells, motivating the continued search for alternative treatments, such as antisense ODNs

that target and impact upon the expression of TS mRNA (U.S. Patent No. 6,087,489; International Patent Applications WO 99/15648 and WO 98/49287). A specific antisense oligonucleotide targeting the 3'-untranslated region of TS mRNA has been shown to down-regulate the expression of TS, inhibit neoplastic cell proliferation (Berg *et al.*, 2001, *J.Pharmacol.Exp.Ther.* **298**:477-484) and enhance the cytotoxicity of certain TS-targeting drugs in HeLa cells (Ferguson, *et al.*, 1999, *Br.J.Pharmacol.* **127**:1777-1786). More recently, the use of this antisense oligonucleotide to increase the sensitivity of cells that over-express TS to 5-FUdR has been demonstrated (Ferguson, *et al.*, 2001, *Br. J. Pharmacol.* **134**:1437-1446).

This background information is provided for the purpose of making known information believed by the applicant to be of possible relevance to the present invention. No admission is necessarily intended, nor should be construed, that any of the preceding information constitutes prior art against the present invention.

SUMMARY OF THE INVENTION

An object of the present invention is to provide antisense oligonucleotides and uses thereof for improving cancer treatment strategies.

In accordance with an aspect of the present invention, there is provided a method of identifying drug targets for cancer therapy comprising: contacting a population of cells that express thymidylate synthase with an antisense oligonucleotide comprising at least 7 consecutive nucleotides complementary to a thymidylate synthase mRNA, wherein said antisense oligonucleotide modulates expression of thymidylate synthase, and identifying genes whose expression is modulated relative to control cells that have not been contacted with said antisense oligonucleotide, wherein said genes, or the gene products thereof, are potential drug targets for cancer therapy.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 illustrates the effect of an antisense oligonucleotide targeting thymidylate synthase (SEQ ID NO:10) on TS gene transcription in HeLa cells *in vitro* as determined by a Run-on transcription assay;

Figure 2A-B illustrates the effect of an antisense oligonucleotide targeting thymidylate synthase (SEQ ID NO:10) on TS mRNA levels in HeLa cells *in vitro* as determined by RT-PCR (A) and real-time PCR (B);

Figure 3 illustrates the effect of an antisense oligonucleotide targeting thymidylate synthase (SEQ ID NO:10) on TS activity in HeLa cells *in vitro* as determined by a [6-^{3}H]-FdUMP-TS binding assay;

Figure 4A-B illustrates the effect of antisense oligonucleotide targeting thymidylate synthase (SEQ ID NO:10) proliferation rate, sensitivity to various concentrations of raltitrexed, or cell cycle distribution of HeLa cells *in vitro*;

Figure 5 depicts TS gene expression in HeLa cells treated with antisense oligonucleotide targeting thymidylate synthase ODN SEQ ID NO: 2 A) Analysis of TS-specific and GAPDH RT-PCR products. B) Quantitative real-time PCR analysis of TS mRNA. C) Western blot analysis of TS protein levels.

Figure 6 depicts scatter graphs showing transcripts with altered expression in HeLa cells treated with antisense oligonucleotide targeting thymidylate synthase ODN SEQ ID NO: 2 (y-axis) compared with ODN SEQ ID NO: 8 (x-axis), for 8 hours (6A) or 24 hours (6B).

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides strategies for improving known cancer therapies and/or developing new cancer therapies. The strategies involve the use of antisense oligonucleotides (ODNs) against thymidylate synthase (TS) that are capable of modulating the expression of TS and at least one other gene in mammalian cells.

The invention thus provides for a method of identifying genes that are, or whose gene products are, potential targets for cancer therapies using antisense oligonucleotides

(ODNs) that are complementary to a portion of a TS gene and that modulate TS expression.

The invention further provides for antisense ODNs against TS gene that can be used in combination with one or more chemotherapeutic agents in the treatment of cancer to enhance the effect of standard doses of the chemotherapeutic agent and/or minimise one or more side-effects associated with the use of the chemotherapeutic agent.

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

The term "antisense oligonucleotide," as used herein, refers to a nucleotide sequence that is complementary to a portion of a TS gene or mRNA. In the context of the present invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid (RNA), deoxyribonucleic acid (DNA), or modified versions or analogues thereof, or RNA or DNA mimetics.

The term "target gene," as used herein, refers to a gene the expression of which is modulated by an antisense oligonucleotide of the invention. In the context of the present invention, a target gene is a "direct target" if an antisense oligonucleotide is designed to be complementary to a region of the gene or mRNA corresponding to the gene, and an "indirect target" if the antisense oligonucleotide has a downstream or non-antisense effect on the expression of the gene. In accordance with the present invention, the direct target gene is the gene encoding thymidylate synthase.

By "modulating the expression" of a target gene it is meant that the antisense oligonucleotide is capable of either increasing or decreasing the level of target gene gene transcription, the level of target gene mRNA, the level of target gene protein, the activity of target gene protein, or a combination thereof, in cells of a particular cell line when compared to untreated cells of the same cell line.

"Gene product," as used herein, refers to both mRNA transcribed from a gene as well as the protein encoded by the gene. The mRNA can be unprocessed or processed to remove introns. The protein can be in an inactive form (such as a pro-protein or prepro-protein form) or it can be in an active, or processed form.

The term "selectively hybridize," as used herein, refers to the ability of a nucleic acid to bind detectably and specifically to a second nucleic acid. Polynucleotides, oligonucleotides and fragments thereof selectively hybridize to direct target nucleic acid strands under hybridization and wash conditions that minimize appreciable amounts of detectable binding to non-specific nucleic acids. High stringency conditions can be used to achieve selective hybridization conditions as known in the art and discussed herein. Typically, hybridization and washing conditions are performed at high stringency according to conventional hybridization procedures. Washing conditions are typically 1-3 x SSC, 0.1-1% SDS, 50-70°C with a change of wash solution after about 5-30 minutes.

The term "corresponds to," as used herein with reference to nucleic acid sequences, refers to a polynucleotide sequence that is identical to all or a portion of a reference polynucleotide sequence. In contradistinction, the term "complementary to" is used herein to mean that the polynucleotide sequence is identical to all or a portion of the complement of a reference polynucleotide sequence. For illustration, the nucleotide sequence "TATAC" corresponds to a reference sequence "TATAC" and is complementary to a reference sequence "GTATA."

The following terms are used herein to describe the sequence relationships between two or more polynucleotides: "reference sequence," "comparison window," "sequence identity," "percentage of sequence identity," and "substantial identity." A "reference sequence" is a defined sequence used as a basis for a sequence comparison; a reference sequence may be a subset of a larger sequence, for example, as a segment of a full-length mRNA or mRNA sequence, or may comprise a complete mRNA or mRNA sequence. Generally, a reference sequence is at least 20 nucleotides in length, but may be at least 25 nucleotides in length, or at least 50 nucleotides in length. Since two polynucleotides may each (1) comprise a sequence (*i.e.* a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) may

further comprise a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity.

A "comparison window," as used herein, refers to a conceptual segment of at least 20 contiguous nucleotide positions wherein a polynucleotide sequence may be compared to a reference sequence of at least 20 contiguous nucleotides and wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (*i.e.* gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith and Waterman (1981) *Adv. Appl. Math.* **2**:482, by the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* **48**:443, by the search for similarity method of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci.* (U.S.A.) **85**:2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 573 Science Dr., Madison, WI), or by inspection. The best alignment (*i.e.* resulting in the highest percentage of identity over the comparison window) generated by the various methods is then selected.

The term "sequence identity" means that two polynucleotide sequences are identical (*i.e.* on a nucleotide-by-nucleotide basis) over the window of comparison. The term "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (*e.g.* A, T, C, G, U, or I) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (*i.e.* the window size), and multiplying the result by 100 to yield the percentage of sequence identity.

The term "substantial identity," as used herein, denotes a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence that has at

least 50 percent sequence identity, and more usually at least 60 percent sequence identity as compared to a reference sequence over a comparison window of at least 20 nucleotide positions, and frequently over a window of at least 25-50 nucleotides, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the polynucleotide sequence which may include deletions or additions which total 20 percent or less of the reference sequence over the window of comparison.

Strategies utilising Antisense Oligonucleotides against Thymidylate Synthase (TS)

The strategies provided by the present invention are based on the observed ability of antisense oligonucleotides (ODNs) that modulate the expression of TS to also modulate the expression of one or more other genes in a cell. As TS is upregulated in most cancer cells and is an important target for cancer chemotherapy, identification of genes (indirect target genes) whose expression is affected by changes in TS expression will provide potential targets for new and/or improved cancer therapeutics.

For example, indirect target genes that are identified using TS antisense ODNs that decrease TS expression are targets for therapeutics designed to bring about the same effect as the TS antisense ODN, *i.e.* up- or down-regulation of expression of the target gene or gene product. Such therapeutics can be used alone or in combination with a TS targeting drug in the treatment of cancer. Alternatively, if therapeutics are currently available that target the identified gene or its gene product, then new combination therapies comprising of TS targeting drugs in combination with a drug that targets the identified gene or its gene product can be developed. The present invention also contemplates that the TS antisense ODNs themselves may be used as TS targeting therapeutic agents in combination with other chemotherapeutics.

Similarly, genes whose expression is modulated in response to treatment with a TS antisense ODN that upregulates TS expression are targets for therapeutics designed to mitigate the effects of increases in TS expression. Moreover, as demonstrated herein certain antisense ODNs that upregulate TS expression can bring about an increase in

TS protein activity without any increase in proliferative ability of the cells. Thus, the upregulatory antisense ODNs themselves, as well as therapeutics designed to modulate expression of the indirect target gene(s) of these antisense ODNs, can be used to suppress the side-effects of TS targeting drugs. Indirect target genes of these latter antisense ODNs can also be used to mitigate the effects of increased TS protein levels or TS protein activity.

The strategies provided by the present invention involve first selecting an appropriate antisense ODN that targets TS. The ability of the antisense ODN to modulate the expression of TS is then determined, followed by an assessment of the up- and/or down-regulation of other genes in cells treated with the antisense ODN and an identification of these genes and the extent to which their expression is modulated by the antisense ODN.

Antisense Oligonucleotides

Selection and characteristics

"Targeting" an antisense ODN to a thymidylate synthase mRNA, in the context of the present invention, is a multistep process. The process usually begins with the identification of a direct target nucleic acid sequence whose function is to be modulated. This may be, for example, a cellular gene or mRNA transcribed from the gene. The targeting process also includes determination of a site or sites within this gene for the antisense interaction to occur such that the desired effect, *i.e.* modulation of expression of the protein encoded by the gene, will result. Once the direct target site or sites have been identified, oligonucleotides are chosen that are sufficiently complementary (*i.e.* hybridize with sufficient strength and specificity) to the direct target mRNA to give the desired result.

The sequences of various TS genes and mRNAs are known in the art and can be readily obtained from Genbank (maintained by the National Center for Biotechnology Information). For example, the sequence for human TS mRNA can be accessed under Accession No. X02308 [SEQ ID NO:1].

Generally, there are five regions of a gene or mRNA that may be targeted for antisense modulation: the 5'-untranslated region (5'-UTR), the translation initiation or start codon region, the open reading frame (ORF), the translation termination or stop codon region and the 3'-untranslated region (3'-UTR). Within these regions, certain sequences may be directly targeted, for example, known regulatory sequence elements (such as those for post-transcriptional control and mRNA stability) or regions that are unique to a group of mRNAs encoding similar proteins.

The terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine in eukaryotes. It is also known in the art that eukaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the invention, "start codon" and "translation initiation codon" refer to the codon or codons that are used *in vivo* to initiate translation of an mRNA molecule transcribed from a gene encoding TS regardless of the sequence(s) of such codons.

As is known in the art, some eukaryotic transcripts are directly translated, however, most mammalian ORFs contain one or more sequences, known as "introns," which are excised from a transcript before it is translated; the expressed (unexcised) portions of the ORF are referred to as "exons" (Alberts *et al.*, (1983) *Molecular Biology of the Cell*, Garland Publishing Inc., New York, pp. 411-415). In the context of the present invention, both introns and exons may serve as targets for antisense modulation.

Thus, the antisense ODNs according to the present invention can be complementary to regions of the unprocessed mRNA of the direct target gene including the introns, or the antisense ODNs can be complementary to part of the processed mRNA of the direct target gene.

In some instances, an ORF may also contain one or more sites that may be directly targeted for antisense modulation due to some functional significance *in vivo*. Examples of the latter types of sites include intragenic stem-loop structures (see, for example, U.S. Patent No. 5,512,438) and, in unprocessed mRNA molecules,

intron/exon splice sites. In addition, mRNA molecules possess a 5'-cap region that may also serve as a direct target for antisense. The 5'-cap of a mRNA comprises an N^7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 5'-cap region of a mRNA is considered to include the 5'-cap structure itself as well as the first 50 nucleotides adjacent to the cap.

There are also elements in the 3'-UTR region which can impact upon message stability, including examples of unique *cis*-elements that interact with trans-acting proteins to control mRNA turnover rates (Hake and Richer, (1997) *Biochim. Biophys. Acta* **1332**:M31-M38). In addition, the polyadenylated tail can serve several functions impacting upon translation efficiency and message turnover, for example, by protecting the message from degradation, depending on the length of the poly 'A' tail (Ford *et al*, (1997) *Mol. Cell. Biol.* **17**:398-406).

In one embodiment of the invention, the antisense ODNs target the 3'-UTR region of a TS mRNA or gene. In another embodiment of the invention, the antisense ODNs target the start codon of a TS mRNA or gene.

The antisense ODNs in accordance with the present invention are selected from a sequence complementary to the direct target gene such that the sequence exhibits the least likelihood of forming duplexes, hair-pins, or of containing homooligomer / sequence repeats. The ODN may further contain a GC clamp. One skilled in the art will appreciate that these properties can be determined qualitatively using various computer modelling programs, for example, the program OLIGO® Primer Analysis Software, Version 5.0 (distributed by National Biosciences, Inc., Plymouth, MN).

Alternatively, the antisense ODNs can be selected on the basis that the sequence is highly conserved for the direct target gene between two or more species. These properties can be determined using the BLASTN program (Altschul, *et al.*, (1990) *J. Mol. Biol.*, **215**:403-10) of the University of Wisconsin Computer group (GCG) software (Devereux. *et al.*, (1984) *Nucleic Acids Res.*, **12**:387-395) with the National Center for Biotechnology Information (NCBI) databases.

In order to be effective, antisense ODNs are typically between 7 and 100 nucleotides in length. In one embodiment of the present invention the antisense ODNs comprise from at least about 7 to about 50 nucleotides, or nucleotide analogues. In another embodiment, the antisense ODNs comprise from about 12 to about 35 nucleotides, or nucleotide analogues. In another embodiment, the antisense ODNs comprise from about 15 to about 25 nucleotides, or nucleotide analogues.

It is understood in the art that an antisense ODN need not have 100% identity with the complement of its direct target sequence. The antisense ODNs in accordance with the present invention have a sequence that is at least about 75% identical to the complement of direct target sequence. In one embodiment of the present invention, the antisense oligonucleotides have a sequence that is at least about 90% identical to the complement of the direct target sequence. In another embodiment, they have a sequence that is at least about 95% identical to the complement of direct target sequence, allowing for gaps or mismatches of several bases. Identity can be determined, for example, by using the BLASTN program of the University of Wisconsin Computer Group (GCG) software.

In accordance with the present invention, the antisense ODNs comprise at least 7 consecutive nucleotides that are complementary to the sequence of a TS mRNA. In one embodiment, the antisense ODNs comprise at least 7 consecutive nucleotides that are complementary to a human TS mRNA. In another embodiment, the antisense ODNs comprise at least 7 consecutive nucleotides that are complementary to the human TS mRNA set forth in SEQ ID NO: 1.

In order for the antisense ODNs of the present invention to function as modulators of the expression of TS mRNA, it is necessary that they demonstrate adequate specificity for the direct target sequence and do not bind to other sequences in the cell. Therefore, in addition to possessing an appropriate level of sequence identity to the complement of a TS mRNA, the antisense ODNs of the present invention should not closely resemble other known sequences. The antisense ODNs of the present invention, therefore, preferably have less than 15 nucleotides identical to any other sequence, more preferably less than 12 nucleotides identical and most preferably less than 7 nucleotides identical to any other sequence.

It will, however, be appreciated by one skilled in the art that the degree of acceptable identity between sequences may vary, for example, according to the length of the antisense ODNs and the relative position of the identical nucleotides in the sequences within a comparison window, such that greater than a 15 nucleotide identity may exist, and the antisense ODN still demonstrates adequate specificity for a direct target sequence. The identity of the antisense ODNs of the present invention to other sequences can be determined, for example, through the use of the BLASTN program and the NCBI databases as indicated above.

In one embodiment of the present invention, the antisense ODNs against TS comprise at least 7 consecutive nucleotides from one of the sequences provided in Table 1. In another embodiment, the antisense ODNs comprise at least 10 consecutive nucleotides from one of the sequences provided in Table 1.

Table 1. Exemplary Antisense ODNs against Human TS

Sequence (5'→ 3')	*Complementary Region in TS mRNA*	SEQ ID NO
GCCAGTGGCAACATCCTTAA	1184-1203	2
TTGGATGCGGATTGTACCCT	1002-1021	3
ACTCAGCTCCCTCAGATTTG	1436-1455	4
CCAGCCCAACCCCTAAAGAC	1081-1100	5
GGCATCCCAGATTTTCACTC	419-438	6
AGCATTTGTGGATCCCTTGA	380-399	7
GCCGGCCACAGGCATGGCGC	Region spanning start codon	10

Modifications to Antisense Oligonucleotides

In the context of this invention, the term "oligonucleotide" includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions, which function similarly. Such modified or substituted oligonucleotides are

often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for the nucleic acid target and increased stability in the presence of nucleases. The term also includes chimeric oligonucleotides. Chimeric oligonucleotides are oligonucleotides that contain two or more chemically distinct regions, each region comprising at least one monomer unit. The oligonucleotides according to the present invention can be single-stranded or they can be double-stranded.

As is known in the art, a nucleoside is a base-sugar combination and a nucleotide is a nucleoside that further includes a phosphate group covalently linked to the sugar portion of the nucleoside. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound, with the normal linkage or backbone of RNA and DNA being a 3' to 5' phosphodiester linkage. Specific examples of oligonucleotides useful in this invention include those containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include both those that retain a phosphorus atom in the backbone and those that lack a phosphorus atom in the backbone. For the purposes of the present invention, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleotides.

Exemplary modified oligonucleotide backbones that can be incorporated into the oligonucleotides according to the present invention include, for example, phosphorothioates, chiral phosphorothioates, bridged phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates, bridged methylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate, bridged phosphoramidates and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogues of these, and analogues having inverted polarity wherein the adjacent pairs of nucleoside units are

linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included.

Exemplary modified oligonucleotide backbones that do not include a phosphorus atom are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. Such backbones include morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulphide, sulphoxide and sulphone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; carbonate backbones; carboxymethylester backbones; acetamidate backbones; carbamate backbones; thioether backbones; alkene containing backbones; sulphamate backbones; methyleneimino and methylenehydrazino backbones; sulphonate and sulphonamide backbones; amide backbones; and others having mixed N, O, S and CH_2 component parts.

The term "alkyl" as used herein refers to monovalent alkyl groups having from 1 to 20 carbon atoms. In one embodiment of the present invention the alkyl group has between 1 and 6 carbon atoms. Examples of suitable alkyl groups include, but are not limited to, methyl, ethyl, *n*-propyl, *iso*-propyl, *n*-butyl, *iso*-butyl, *n*-hexyl, and the like.

The term "cycloalkyl" refers to cyclic alkyl groups of from 3 to 20 carbon atoms having a single cyclic ring or multiple condensed rings. Examples of suitable cycloalkyl groups include, but are not limited to, single ring structures such as cyclopropyl, cyclobutyl, cyclopentyl, cyclooctyl, and the like, or multiple ring structures such as adamantanyl, and the like.

The present invention also contemplates oligonucleotide mimetics in which both the sugar and the internucleoside linkage of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target. An example of such an oligonucleotide mimetic, which has been shown to have excellent hybridization properties, is a peptide nucleic acid (PNA) [Nielsen *et al.*, *Science*, **254**:1497-1500 (1991)]. In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide-containing backbone, in particular an

aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza-nitrogen atoms of the amide portion of the backbone.

The present invention also contemplates oligonucleotides comprising "locked nucleic acids" (LNAs), which are novel conformationally restricted oligonucleotide analogues containing a methylene bridge that connects the 2'-O of ribose with the 4'-C (see, Singh *et al., Chem. Commun.*, 1998, 4:455-456). LNA and LNA analogues display very high duplex thermal stabilities with complementary DNA and RNA, stability towards 3'-exonuclease degradation, and good solubility properties. Synthesis of the LNA analogues of adenine, cytosine, guanine, 5-methylcytosine, thymine and uracil, their oligomerization, and nucleic acid recognition properties have been described (see Koshkin *et al., Tetrahedron,* 1998, 54:3607-3630). Studies of mis-matched sequences show that LNA obey the Watson-Crick base pairing rules with generally improved selectivity compared to the corresponding unmodified reference strands. Antisense oligonucleotides containing LNAs have been described (Wahlestedt *et al., Proc. Natl. Acad. Sci. U. S. A.*, 2000, 97:5633-5638), which were efficacious and non-toxic. In addition, the LNA/DNA copolymers were not degraded readily in blood serum and cell extracts.

LNAs form duplexes with complementary DNA or RNA or with complementary LNA, with high thermal affinities. The universality of LNA-mediated hybridization has been emphasized by the formation of exceedingly stable LNA:LNA duplexes (Koshkin *et al., J. Am. Chem. Soc.*, 1998, 120:13252-13253). LNA:LNA hybridization was shown to be the most thermally stable nucleic acid type duplex system, and the RNA-mimicking character of LNA was established at the duplex level. Introduction of three LNA monomers (T or A) resulted in significantly increased melting points toward DNA complements.

Synthesis of 2'-amino-LNA (Singh et al., J. Org. Chem., 1998, 63, 10035-10039) and 2'-methylamino-LNA has been described and thermal stability of their duplexes with complementary RNA and DNA strands reported. Preparation of phosphorothioate-LNA and 2'-thio-LNA have also been described (Kumar *et al., Bioorg. Med. Chem. Lett.*, 1998, 8:2219-2222).

Modified oligonucleotides according to the present invention may also contain one or more substituted sugar moieties. For example, oligonucleotides may comprise sugars with one of the following substituents at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C_1 to C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl. Examples of such groups are: $O[(CH_2)_n O]_m CH_3$, $O(CH_2)_n OCH_3$, $O(CH_2)_n NH_2$, $O(CH_2)_n CH_3$, $O(CH_2)_n ONH_2$, and $O(CH_2)_n ON[(CH_2)_n CH_3)]_2$, where n and m are from 1 to about 10. Alternatively, the oligonucleotides may comprise one of the following substituents at the 2' position: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, $SO_2 CH_3$, ONO_2, NO_2, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. Specific examples include 2'-methoxyethoxy (2'-O-CH_2 CH_2 OCH_3, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) [Martin *et al.*, *Helv. Chim. Acta,* **78**:486-504(1995)], 2'-dimethylaminooxyethoxy ($O(CH_2)_2$ $ON(CH_3)_2$ group, also known as 2'-DMAOE), 2'-methoxy (2'-O-CH_3), 2'-aminopropoxy (2'-OCH_2 CH_2 CH_2 NH_2) and 2'-fluoro (2'-F).

Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of the 5' terminal nucleotide. Oligonucleotides may also comprise sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar.

Oligonucleotides according to the present invention may also include modifications or substitutions to the nucleobase. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C); inosine; 5-hydroxymethyl cytosine; xanthine; hypoxanthine; 2-aminoadenine; 6-methyl and other alkyl derivatives of adenine and guanine; 2-propyl and other alkyl derivatives of

adenine and guanine; 2-thiouracil, 2-thiothymine and 2-thiocytosine; 5-halouracil and cytosine; 5-propynyl uracil and cytosine; 6-azo uracil, cytosine and thymine; 5-uracil (pseudouracil); 4-thiouracil; 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines; 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines; 7-methylguanine and 7-methyladenine; 8-azaguanine and 8-azaadenine; 7-deazaguanine and 7-deazaadenine; 3-deazaguanine and 3-deazaadenine. Further nucleobases include those disclosed in U.S. Pat. No. 3,687,808; The Concise Encyclopaedia Of Polymer Science And Engineering, (1990) pp 858-859, Kroschwitz, J. I., ed. John Wiley & Sons; Englisch *et al.*, *Angewandte Chemie, Int. Ed.*, **30**:613 (1991); and Sanghvi, Y. S., (1993) *Antisense Research and Applications*, pp 289-302, Crooke, S. T. and Lebleu, B., ed., CRC Press. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6 – 1.2°C [Sanghvi, Y. S., (1993) *Antisense Research and Applications*, pp 276-278, Crooke, S. T. and Lebleu, B., ed., CRC Press, Boca Raton].

Another oligonucleotide modification included in the present invention is the chemical linkage to the oligonucleotide of one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. Such moieties include, but are not limited to, lipophilic or lipid moieties such as a cholesterol moiety [Letsinger *et al.*, *Proc. Natl. Acad. Sci. USA*, **86**:6553-6556 (1989)], cholic acid [Manoharan *et al.*, *Bioorg. Med. Chem. Let.*, **4**:1053-1060 (1994)], a thioether, *e.g.* hexyl-S-tritylthiol [Manoharan *et al.*, *Ann. N.Y. Acad. Sci.*, **660**:306-309 (1992); Manoharan *et al.*, *Bioorg. Med. Chem. Lett.*, **3**:2765-2770 (1993)], a thiocholesterol [Oberhauser *et al.*, *Nucl. Acids Res.*, **20**:533-538 (1992)], an aliphatic chain, *e.g.* dodecandiol or undecyl residues [Saison-Behmoaras *et al.*, *EMBO J.*, **10**:1111-1118 (1991); Kabanov *et al.*, *FEBS Lett.*, **259**:327-330 (1990); Svinarchuk *et al.*, *Biochimie*, **75**:49-54 (1993)], a phospholipid, *e.g.* di-hexadecyl-*rac*-glycerol or triethylammonium 1,2-di-O-hexadecyl-*rac*-glycero-3-H-phosphonate [Manoharan *et al.*, *Tetrahedron Lett.*, **36**:3651-3654 (1995); Shea *et al.*, *Nucl. Acids Res.*, **18**:3777-

3783 (1990)], a polyamine or a polyethylene glycol chain [Manoharan *et al.*, *Nucleosides & Nucleotides,* **14**:969-973 (1995)], or adamantane acetic acid [Manoharan *et al.*, *Tetrahedron Lett.,* **36**:3651-3654 (1995)], a palmityl moiety [Mishra *et al.*, *Biochim. Biophys. Acta,* **1264**:229-237 (1995)], or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety [Crooke *et al.*, *J. Pharmacol. Exp. Ther.*, **277**:923-937 (1996)].

One skilled in the art will recognise that it is not necessary for all positions in a given oligonucleotide to be uniformly modified. The present invention, therefore, contemplates the incorporation of more than one of the aforementioned modifications into a single oligonucleotide or even at a single nucleoside within the oligonucleotide.

As indicated above, oligonucleotides that are chimeric compounds are included within the scope of the present invention. For purposes of the invention, a "chimeric oligonucleotide" refers to an oligonucleotide having more than one type of internucleoside linkage or comprising more than one type of modified nucleotide. Non-limiting examples include oligonucleotides having an alkylphosphonate-linked region and an alkylphosphonothioate region (as described; for example, in U.S. Patent Nos. 5,635,377 and 5,366,878), oligonucleotides containing at least one, or more typically, at least three or four consecutive phosphodiester or phosphorothioate internucleoside linkages. Other examples of chimeric oligonucleotides include those comprising a ribonucleotide or 2′-O-substituted ribonucleotide region (for example, comprising from about 2 to about 12 2′-O-substituted nucleotides), and a deoxyribonucleotide region. Such chimeric oligonucleotides have been previously described (see, for example, U.S. Patent Nos. 5,652,355 and 5,652,356). Inverted chimeric oligonucleotides are also contemplated, as described in U.S. Patent Nos. 5,652,356; 5,973,136, and 5,773,601.

Particularly useful chimeric oligonucleotides are mixed backbone oligonucleotides (MBOs) which contain centrally-modified or end-modified nucleosides with appropriately placed segments of modified internucleotide linkages, such as phosphorothioates, methylphosphonates, phosphodiesters, and segments of modified oligodeoxynucleotides or oligoribonucleotides (Agrawal (1997) *Proc. Natl. Acad. Sci., USA,* 94:2620-2625; Agrawal (1999) *Biochem. Biophys. Acta* 1489:53-67).

In the context of the present invention, an oligonucleotide is "nuclease resistant" when it has either been modified such that it is not susceptible to degradation by DNA and RNA nucleases or, alternatively, has been placed in a delivery vehicle which itself protects the oligonucleotide from DNA or RNA nucleases. Nuclease resistant oligonucleotides include, for example, methyl phosphonates, phosphorothioates, phosphorodithioates, phosphotriesters, and morpholino oligomers. Suitable delivery vehicles for conferring nuclease resistance include, for example, liposomes.

The present invention further contemplates oligonucleotides that contain groups for improving the pharmacokinetic properties of the oligonucleotide, or groups for improving the pharmacodynamic properties of the oligonucleotide.

In one embodiment of the present invention, the antisense ODNs comprise at least one phosphorothioate linkage. In another embodiment, the antisense ODNs comprise at least one 2′-methoxy-ethoxy substituted nucleotide. In another embodiment, the antisense ODNs comprise one or more 2′-methoxy-ethoxy substituted nucleotides at both the 3′- and 5′-end of the ODN. In still another embodiment, the antisense ODNs comprise both at least one phosphorothioate linkage and one or more 2′-methoxy-ethoxy substituted nucleotides at both the 3′- and 5′-ends of the ODN.

Short Interfering RNA (siRNA) Molecules

The present invention further contemplates that the antisense ODNs may be in the form of siRNA molecules. RNA interference mediated by double-stranded siRNA molecules, which are generated in nature when long double-stranded RNA molecules are cleaved by the action of an endogenous ribonuclease, is known in the art to play an important role in post-transcriptional gene silencing [Zamore, *Nature Struc. Biol.*, **8**:746-750 (2001)]. Transfection of mammalian cells with synthetic siRNA molecules having a sequence identical to a target gene has been demonstrated to result in a reduction in the mRNA levels of the target gene [see, for example, Elbashir, *et al.*, *Nature*, **411**:494–498 (2001)]. siRNA molecules are typically 21–22 base pairs in length.

The specificity of siRNA molecules is determined by the binding of the antisense strand of the molecule to its target mRNA. Thus, the antisense ODNs of the present invention can be provided as siRNA molecules which are targeted to a TS gene. As is known in the art, effective siRNA molecules should be less than 30 to 35 base pairs in length to prevent them triggering non-specific RNA interference pathways in the cell via the interferon response. Thus, in one embodiment of the present invention, the siRNA molecules are between about 15 and about 25 base pairs in length. In a related embodiment, they are between 19 and 22 base pairs in length.

The double-stranded siRNA molecules can further comprise poly-T or poly-U overhangs at each end to minimise RNase-mediated degradation of the molecules. In another embodiment of the present invention, the siRNA molecules comprise overhangs at the 3′ and 5′ ends which consist of two thymidine or two uridine residues. Design and construction of siRNA molecules is known in the art [see, for example, Elbashir, *et al., Nature*, **411**:494–498 (2001); Bitko and Barik, *BMC Microbiol.*, **1**:34 (2001)]. In addition, kits that provide a rapid and efficient means of constructing siRNA molecules by *in vitro* transcription are also commercially available (Ambion, Austin, TX; New England Biolabs, Beverly, MA).

Single-stranded siRNA and short-hairpin siRNA (shRNA) molecules are also known in the art. The present invention contemplates that the antisense ODNs against TS can be provided as single-stranded siRNA molecules and as shRNA molecules.

Preparation of the Antisense Oligonucleotides

The antisense ODNs of the present invention can be prepared by conventional techniques well-known to those skilled in the art (see, for example, U.S. Patent No. 6,087,489). For example, the oligonucleotides can be prepared using solid-phase synthesis using commercially available equipment, such as the equipment available from Applied Biosystems Canada Inc., Mississauga, Canada. As is well-known in the art, modified oligonucleotides, such as phosphorothioates and alkylated derivatives, can also be readily prepared by similar methods.

The isolation and purification of antisense oligonucleotides can be accomplished using, for example, filtration, extraction, crystallization, different forms of chromatography, including column, thin layer, preparative low or high pressure liquid chromatography, or a combination of these procedures, in addition to other equivalent separation or isolation procedures.

Alternatively, the antisense oligonucleotides of the present invention can be prepared by enzymatic digestion of the naturally occurring direct target gene by methods known in the art.

Antisense oligonucleotides can also be prepared through the use of recombinant methods. The present invention, therefore encompasses expression vectors comprising nucleic acid sequences that encode the antisense oligonucleotides and expression of the encoded antisense oligonucleotides in a suitable host cell. Such expression vectors can be readily constructed using procedures known in the art. Examples of suitable vectors include, but are not limited to, plasmids, phagemids, cosmids, bacteriophages, baculoviruses and retroviruses, and DNA viruses. One skilled in the art will understand that selection of the appropriate host cell for expression of the antisense oligonucleotide will be dependent upon the vector chosen. Examples of host cells include, but are not limited to, bacterial, yeast, insect, plant and mammalian cells.

One skilled in the art will also understand that the expression vector may further include regulatory elements, such as transcriptional elements, required for efficient transcription of the antisense oligonucleotide sequences. Examples of regulatory elements that can be incorporated into the vector include, but are not limited to, promoters, enhancers, terminators, and polyadenylation signals. The present invention, therefore, provides vectors comprising a regulatory element operatively linked to a nucleic acid sequence encoding an antisense oligonucleotide. One skilled in the art will appreciate that selection of suitable regulatory elements is dependent on the host cell chosen for expression of the antisense oligonucleotide and that such regulatory elements may be derived from a variety of sources, including bacterial, fungal, viral, mammalian or insect genes.

In the context of the present invention, the expression vector may additionally contain a reporter gene. Suitable reporter genes include, but are not limited to, β-galactosidase, green fluorescent protein, red fluorescent protein, luciferase, and β-glucuronidase. Incorporation of a reporter gene into the expression vector allows transcription of the antisense oligonucleotide to be monitored by detection of a signal generated by expression of the reporter gene.

In accordance with the present invention, the expression vectors can be introduced into a suitable host cell or tissue by one of a variety of methods known in the art. Such methods can be found generally described in Sambrook *et al., Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York (1992); Ausubel *et al., Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore Maryland (1989); Chang *et al., Somatic Gene Therapy*, CRC Press, Ann Arbor MI (1995); Vega *et al., Gene Targeting*, CRC Press, Ann Arbor, MI (1995); and Vectors: A Survey of Molecular Cloning Vectors and Their Uses, Butterworth's, Boston MA (1988) and include, for example, stable or transient transfection, lipofection, electroporation, and infection with recombinant viral vectors.

Testing of the Antisense Oligonucleotides

In accordance with the present invention, the antisense ODNs are selected for their ability to modulate TS expression and for their ability to modulate the expression of at least one other indirect target gene.

The effectiveness of the antisense ODNs of the present invention in modulating TS expression can be demonstrated initially *in vitro* using, for example, the method previously described by Choy *et al.*,1998, *Cancer Res.* **48**: 6949-6952.

For example, the antisense ODN can be introduced, for example by transfection, into a cell line that normally expresses TS or which over-expresses TS (for example, a tumour cell line) and the amount of mRNA transcribed from the TS gene can be measured by standard techniques such as Northern blot analysis, RT-PCR, run-on transcription assays and the like. Alternatively, the amount of TS protein produced by the cell can be measured by standard techniques such as Western blot analysis. The

amount of mRNA or protein produced in a cell treated with the antisense ODN can then be compared with the amount produced in control cells and will provide an indication of whether the antisense ODN has modulated TS gene expression. Suitable control cells include untreated cells and cells treated with a control, scrambled ODN.

Antisense ODNs in accordance with the invention are those ODNs that modulate TS expression, *i.e.* either increase or decrease, the level of TS gene transcription, the level of TS mRNA, the level of TS protein or the activity of TS protein, when compared to untreated cells, or cells treated with a control oligonucleotide. In one embodiment of the present invention, the antisense ODN modulates the expression of TS by at least 10% compared to an untreated control. In another embodiment, the antisense oligonucleotide modulates TS expression by increasing the level of TS gene transcription, the level of TS mRNA, the level of TS protein, the activity of TS protein, or a combination thereof. In an alternative embodiment, the antisense oligonucleotide modulates TS expression by decreasing the level of TS gene transcription, the level of TS mRNA, the level of TS protein, the activity of TS protein, or a combination thereof.

The antisense ODNs of the present invention can be screened for their ability to modulate the expression of genes other than the TS gene using standard methods for screening expression of multiple genes ("expression profiling"). Such methods are well known in the art and include, for example, the microarray assay described in the Examples, or high density microarray assays containing 10-fold more (for example, 19,000) human genes to identify suitable functional clusters of genes whose expression is affected by the antisense ODNs.

Typically, expression profiling makes use of pre-fabricated microarrays of short DNA sequences or oligonucleotides. Microarrays comprise an ordered arrangement of thousands of oligonucleotides, each capable of specifically hybridising to a certain gene, immobilised onto a suitable solid support. Typically microarrays useful for this purpose represent between 1,000 and 40,000 genes. Methods of constructing microarrays are well known in the art [see, for example, Ausubel, *et al., Current Protocols in Molecular Biology,* John Wiley & Sons, Inc, NY. (1989 and updates)]. In addition, custom-made microarrays are available from many companies. Pre-made

microarrays are also commercially available for many organisms including, for example, GeneChip® (Affimetrix, Santa Clara, CA), Atlas™ (BD Biosciences-CLONTECH, Palo Alto, CA), GEM Microarrays, GeneJet™ array and LifeSeq® (Incyte Genomics, Palo Alto, CA), MICROMAX™ Human cDNA Microarray Systems (PerkinElmer Life Sciences, Boston, Mass.) and ResGen™ GeneFilters® (Invitrogen, Huntsville, Ala.).

For expression analysis, RNA is isolated from cells treated with the antisense ODN and from control cells. If necessary, the RNA can be amplified by conventional techniques to ensure a sufficient quantity for analysis. The RNA is then hybridised to the microarray under suitable conditions and a routine analysis of the microarray by commercially available scanners and software is conducted to identify genes whose expression is altered in the treated cells relative to the control cells. Suitable hybridization conditions can readily be determined by one skilled in the art using standard techniques.

Following the identification of such indirect target genes, the extent of modulation of expression of individual genes can be assessed, for example mRNA quantitation and/or respective protein levels can be evaluated using techniques such as those described above for TS.

Indirect Target Genes

Indirect target genes thus identified are useful therapeutic targets. Examples of indirect target genes identified using antisense ODNs of the invention, which may be suitable therapeutic targets are outlined in Tables 3-6 (provided herein in the Examples section).

When an identified indirect target gene is already known to play a role in cancer and/or act as a target for known chemotherapeutics, then the identification of its inter-relation with TS using the strategies of the present invention can allow new combination therapies to be developed that include, for example, combinations of chemotherapeutics targeting different indirect targets or combinations of a chemotherapeutic targeting an indirect target and a chemotherapeutic targeting TS.

Combinations that include the antisense ODNs of the invention as a therapeutic agent are also contemplated.

Anticancer Activity

If desired, the ability of the antisense ODNs, alone or in combination with a known chemotherapeutic, to inhibit cancer cell growth can be tested *in vitro* and/or *in vivo*. For example, the colony forming ability or proliferation of neoplastic cells treated with the antisense ODNs can be tested by growing neoplastic cells to an appropriate density (e.g. approximately 1 x 10^4) and then adding an appropriate concentration of one or more antisense ODN in the presence of cationic lipid (e.g. lipofectin to a final concentration of 5 μg/mL). Excess antisense ODN is washed away after a suitable incubation period and the cells are subsequently cultured using standard techniques. Percent inhibition of colony forming ability or proliferation is calculated by comparison of the number of colonies in the treated culture with the number of colonies in control cultures, for example, cultures not pre-treated with antisense ODNs, those pre-treated with a control, scrambled ODN or those treated with a standard chemotherapeutic (as visualized, for example, by methylene blue staining). Similar methods can be employed to determine the effect of the antisense ODNs in combination with one or more chemotherapeutic.

The efficacy of the antisense ODNs of the present invention can be tested *in vivo* in an appropriate animal model. In general, current animal models for screening anti-tumour compounds are xenograft models, in which a human tumour has been implanted into an animal. Examples of xenograft models of human cancer include, but are not limited to, human solid tumour xenografts in mice, implanted by sub-cutaneous injection and used in tumour growth assays; human solid tumour isografts in mice, implanted by fat pad injection and used in tumour growth assays; experimental models of lymphoma and leukaemia in mice, used in survival assays; and experimental models of lung metastasis in mice. Examples of currently accepted xenograft models are provided in Table 2.

Antisense ODNs alone or in combination with one or more chemotherapeutic may be administered to neoplastic cells *ex vivo* prior to injection of the cells into the mice or

they may be administered to the mice *in vivo* after the injection of the cells and tumour establishment in the mice.

When administered *in vivo*, the antisense oligonucleotides of the present invention can be administered to the animal by, for example, systemic administration (e.g. tail vein injection) or local administration, *e.g.* into a tumour. Alternatively, the antisense ODNs can be administered by continuous spinal delivery, for example, via an intratheal catheter attached to a mini-osmotic pump.

As an example, the antisense ODN in combination with one or more chemotherapeutic can be tested *in vivo* on solid tumours using mice that are subcutaneously grafted bilaterally with a pre-determined amount of a tumour fragment on day 0. Orthotopically implanted tumours may also be used. The animals bearing tumours are mixed before being subjected to the various treatments and controls. In the case of treatment of advanced tumours, tumours are allowed to develop to the desired size, animals having insufficiently developed tumours being eliminated. The selected animals are distributed at random into groups that will undergo the treatments or act as controls. Suitable groupings would be, for example, those receiving the antisense ODN and the one or more chemotherapeutic, those receiving the antisense ODN alone, those receiving the chemotherapeutic agent(s) alone and those receiving no treatment or treatment with a control, scrambled ODN. Animals not bearing tumours may also be subjected to the same treatments as the tumour-bearing animals in order to be able to dissociate the toxic effect from the specific effect on the tumour. Chemotherapy generally begins from 3 to 22 days after grafting, depending on the type of tumour, and the animals are observed every day. The antisense ODNs of the present invention can be administered to the animals, for example, by bolus infusion. The different animal groups are weighed about 3 or 4 times a week until the maximum weight loss is attained, after which the groups are weighed at least once a week until the end of the trial.

The tumours are measured about 2 or 3 times a week until the tumour reaches a pre-determined size and / or weight, or until the animal dies if this occurs before the tumour reaches the pre-determined size / weight. The animals are then sacrificed and the tissue histology, size and / or proliferation of the tumour assessed.

For the study of the effect of the antisense ODN with one or more chemotherapeutic on leukaemias, the animals are grafted with a particular number of cells, and the anti-tumour activity is determined by the increase in the survival time of the treated mice relative to the controls.

To study the effect of the antisense ODN with one or more chemotherapeutic on tumour metastasis, tumour cells are typically treated *ex vivo* and then injected into a suitable test animal. The spread of the tumour cells from the site of injection is then monitored over a suitable period of time by standard techniques.

In vivo toxic effects of the oligonucleotides can be evaluated by measuring their effect on animal body weight during treatment and by performing haematological profiles and liver enzyme analysis after the animal has been sacrificed.

Table 2: Examples of Xenograft Models of Human Cancer

Cancer Model	***Cell Type***
Tumour Growth Assay	Prostate (PC-3, DU145)
Human solid tumour xenografts in mice (sub-cutaneous injection)	Breast (MDA-MB-231, MVB-9)
	Colon (HT-29)
	Lung (NCI-H460, NCI-H209)
	Pancreatic (ASPC-1, SU86.86)
	Pancreatic: drug resistant (BxPC-3)
	Skin (A2058, C8161)
	Cervical (SIHA, HeLa-S3)
	Cervical: drug resistant (HeLa S3-HU-resistance)
	Liver (HepG2)
	Brain (U87-MG)
	Renal (Caki-1, A498)
	Ovary (SK-OV-3)

Cancer Model	*Cell Type*
Tumour Growth Assay Human solid tumour isografts in mice (fat pad injection)	Breast: drug resistant (MDA-CDDP-S4, MDA-MB435-To.1)
Survival Assay Experimental model of lymphoma and leukaemia in mice	Human: Burkitts lymphoma (Non-Hodgkin's) (raji) Murine: erythroleukemia (CB7 Friend retrovirus-induced)
Experimental model of lung metastasis in mice	Human: melanoma (C8161) Murine: fibrosarcoma (R3)

Studies to assess the toxicology, pharmacokinetics and bioavailability of chemotherapeutics when administered in combination with the antisense ODNs of the present invention can also be conducted using techniques known on the art (see, for example, Maines *et al.*, (eds.) *Current Protocols in Toxicology*, J. Wiley & Sons, New York, NY; "*Pharmacokinetics Processes and Mathematics*", Welling, P.E., ACS Monograph 185; 1986).

Applications for the Antisense Oligonucleotides

As described above, the antisense ODNs of the present invention can be used to identify potential targets for cancer therapies. Treatment of cells with the antisense ODNs and subsequent expression analysis using techniques such as those described above, can provide information about indirect target genes whose expression is affected by the antisense ODNs. Such indirect target genes are potential targets in the development of new chemotherapeutics or cancer treatment strategies.

In addition, the antisense ODNs to TS themselves can also be used to help to improve cancer therapies. For example, the antisense ODNs can be used to sensitise tumour cells to the action of one or more chemotherapeutic agents (*i.e.* enhance the effects of standard dosages of the chemotherapeutics). In particular, the antisense ODNs can be used to sensitise tumour cells to the action of one or more chemotherapeutics that

target either, or both, of TS and the indirect target. Alternatively, the antisense ODNs can be used to sensitise tumour cells to the action of one or more chemotherapeutics that are normally broken down or catabolised *in vivo* by the protein encoded by indirect target gene. Such antisense ODNs can also be used to increase the bioavailability of the one or more chemotherapeutic.

The antisense ODNs of the invention may also be used to decrease the effective dosage of a particular chemotherapeutic agent that needs to be administered to a patient during a chemotherapy regimen. Alternatively, or in addition, the antisense ODNs may be used to decrease the side effects of a chemotherapeutic agent.

Suitable chemotherapeutic agents that target TS include, but are not limited to, the fluoropyrimidine drugs 5-FU, 5-FUdR, capecitabine (an oral form of a pro-drug of 5-FU) and a topical 5-FU cream (Effudex®), as well as the non-fluoropyrimidine drugs raltitrexed, methotrexate and Alimta® (pemetrexed).

The strategies provided by the present invention can be used to improve or develop treatment regimes for various cancers irrespective of their origin. Cancers which may be treated using the methods of the invention, include, but are not limited to carcinomas, leukemias (*e.g.* of the central-nervous system and blood), lymphomas (Hodgkins and non-Hodgkins), sarcomas, melanomas, adenomas, neuroblastomas, nephroblastomas (*e.g.* Wilm's tumour), mesotheliomas and retinoblastomas.

Examples of carcinomas (*i.e.* cancers originating in epithelial tissues such as the skin and inner membrane surfaces of the body), include, but are not limited to cancers such as breast cancer, colon cancer, rectal cancer, esophageal cancer, prostate cancer, lung cancer, stomach cancer, bladder cancer, skin cancer, kidney cancer, pancreatic cancer, ovarian cancer, uterine cancer, cervical cancer, cancer of the vulva, liver cancer, thyroid cancer, aveolar cell carcinoma, basal cell carcinoma, bronchogenic carcinoma, chorionic carcinoma, embyronal carcinoma, giant cell carcinoma, glandular carcinoma, medullary carcinoma, melatonic carcinoma, mucinous carcinoma, oat cell carcinoma, scirrhous carcinoma and squamous cell carcinoma.

Examples of sarcomas (*i.e.* cancers originating in soft tissues of mesenchymal origin such as the connective and supportive tissues of muscle, bone, cartilage and fat),

include, but are not limited to cancers such as Kaposi's sarcoma, alveolar soft part sarcoma, bone cancer, botryoid sarcoma, endometrial sarcoma, giant cell sarcoma, osteogenic sarcoma, reticulum cell sarcoma and spindle cell sarcoma, rhabdomyosarcoma and lymphosarcoma.

Pharmaceutical Preparations Antisense Oligonucleotides

When employed as pharmaceuticals, the antisense oligonucleotides are usually administered in the form of pharmaceutical compositions or formulations. Such compositions are prepared in a manner well known in the pharmaceutical art and comprise at least one active compound. In a related embodiment, the pharmaceutical composition or formulation comprises a vector encoding the antisense oligonucleotide of the present invention.

In accordance with the present invention, the antisense oligonucleotides may be incorporated into pharmaceutical compositions in the form of pharmaceutically acceptable salts. The term "pharmaceutically acceptable salt" as used herein refers to salts which retain the biological effectiveness and properties of the antisense oligonucleotides of the present invention, and which are not biologically or otherwise undesirable. In many cases, the antisense oligonucleotides of the present invention are capable of forming acid and/or base addition salts by virtue of the presence of amino and/or carboxyl groups or groups similar thereto.

Pharmaceutically acceptable base addition salts can be prepared from inorganic and organic bases. Salts derived from inorganic bases include, but are not limited to, sodium, potassium, lithium, ammonium, calcium and magnesium salts. Salts derived from organic bases include, but are not limited to, salts of primary, secondary and tertiary amines, such as alkyl amines, dialkyl amines, trialkyl amines, substituted alkyl amines, di(substituted alkyl) amines, tri(substituted alkyl) amines, alkenyl amines, dialkenyl amines, trialkenyl amines, substituted alkenyl amines, di(substituted alkenyl) amines, tri(substituted alkenyl) amines, cycloalkyl amines, di(cycloalkyl) amines, tri(cycloalkyl) amines, substituted cycloalkyl amines, disubstituted cycloalkyl amine, trisubstituted cycloalkyl amines, cycloalkenyl amines, di(cycloalkenyl) amines, tri(cycloalkenyl) amines, substituted cycloalkenyl amines, disubstituted cycloalkenyl

amine, trisubstituted cycloalkenyl amines, aryl amines, diaryl amines, triaryl amines, heteroaryl amines, diheteroaryl amines, triheteroaryl amines, heterocyclic amines, diheterocyclic amines, triheterocyclic amines, mixed di- and tri-amines where at least two of the substituents on the amine are different and are selected from the group consisting of alkyl, substituted alkyl, alkenyl, substituted alkenyl, cycloalkyl, substituted cycloalkyl, cycloalkenyl, substituted cycloalkenyl, aryl, heteroaryl, heterocyclic, and the like. Amines in which two or three substituents, together with the amino nitrogen, form a heterocyclic or heteroaryl group are also suitable.

Examples of suitable amines include, but are not limited to, isopropylamine, trimethyl amine, diethyl amine, tri(*iso*-propyl) amine, tri(*n*-propyl) amine, ethanolamine, 2-dimethylaminoethanol, tromethamine, lysine, arginine, histidine, caffeine, procaine, hydrabamine, choline, betaine, ethylenediamine, glucosamine, N-alkylglucamines, theobromine, purines, piperazine, piperidine, morpholine, N-ethylpiperidine, and the like. It should also be understood that carboxylic acid derivatives would be useful in the practice of this invention, for example, carboxylic acid amides, including carboxamides, lower alkyl carboxamides, dialkyl carboxamides, and the like.

Pharmaceutically acceptable acid addition salts can be prepared from inorganic and organic acids. Salts derived from inorganic acids include hydrochloric acid, hydrobromic acid, sulphuric acid, nitric acid, phosphoric acid, and the like. Salts derived from organic acids include acetic acid, propionic acid, glycolic acid, pyruvic acid, oxalic acid, malic acid, malonic acid, succinic acid, maleic acid, fumaric acid, tartaric acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, methanesulphonic acid, ethanesulphonic acid, *p*-toluene-sulphonic acid, salicylic acid, and the like.

To gain a better understanding of the invention described herein, the following examples are set forth. It should be understood that these examples are for illustrative purposes only. Therefore, they should not limit the scope of this invention in any way.

EXAMPLES

EXAMPLE 1: Microarray analysis

Oligodeoxynucleotides

A fully phosphorothioated ODN with 2'-methoxy-ethoxy modification on the 6 nucleotides at both the 5'- and 3'-ends was used in the following example: ODN SEQ ID NO: 10 (5'-GCCGGCCACAGGCATGGCGC-3').

The scrambled control ODN SEQ ID NO: 9 has the same base composition as ODN SEQ ID NO: 10, in random order.

Methods

1.1 **Cell Culture:** HeLa cells were grown in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum in 5% CO_2 at 37°C. HeLa cells were plated at 1 $X10^5$ cells/25cm^3 flask for growth and drug sensitivity assays and at $2X10^6$ cells/75 cm^3 flask for mRNA and protein assays. Cells were treated with either the control (SEQ ID NO:9) or antisense (SEQ ID NO:10) ODN, using Lipofectamine 2000 (a cationic liposome) to enhance ODN uptake. For growth and drug sensitivity assays, 50nM ODN with 0.5g/mL Lipofectamine 2000 was used, while for run on transcription, microarray analysis and protein assays, 100nM ODN and 1μg/mL Lipofectamine 2000 was used.

1.2 **Run-On-Transcription Measurement:** HeLa cells are treated with the ODN /liposome solution and left for 24 hours. Nuclei were isolated and run-on-transcription was carried out after addition of reaction buffer, dGTP, dATP, dUTP, 32P-dCTP, and 1mM DTT for 30 minutes at 37°C. Radiolabelled RNA was isolated following TRIzol protocol. Probes specific for TS, GAPDH and 18S were immobilized on a Highland-N membrane and prehybridized for 48 hours and then developed and quantitated using Phosphorimager and Imagequant software.

1.3 **mRNA Measurement:** HeLa Cells were treated with the ODN/Liposome solution and harvested after 24 hours. RNA was isolated using TRIzol, followed by a reverse-transcriptase polymerase chain reaction (RT-PCT) using primers specific for TS and GAPDH. The PCR products were separated by electrophoresis on a 1% agarose gel. SEQ ID NO:8 and SEQ ID NO:2 (scrambled control) was used as a positive control (Ferguson *et al.*(1999), Br. J. Pharm. 127:

1777-86). For real-time PCR,transfection and RNA isolation followed the same protocol. Real time PCR was carried out with TaqMan probes specific for TS and 18S and run on ABI prism 7700.

1.4 **Protein activity levels:** HeLa cells were treated with the ODN/liposome solution and cell lysates were prepared after 24 hours. TS protein was quantitated using a 3 [6- H]-FdUMP binding assay, as described by Ferguson, P. *et al.* Br. J. Pharm. 127: 1777-86, 1999.

1.5 **Growth assay/ Drug sensitivity:** HeLa cells were plated, left overnight and then treated with the ODN/liposome solution in 2 mL of medium for four hours. After this time, 2 mL of medium was added and cells in 3 flasks for each condition were counted each day for four days, by numeration with an electronic particle counter. For drug sensitivity assays, the drug was added 4 hours after treatment with the ODN. Cells were cultured for four days and then counted.

1.6 **Microarray analysis:** HeLa cells were treated with the ODN/liposome solution and left for 24 hours. Cells were harvested, RNA isolated using RNeasy protocol and sent to the London Regional Genomics Centre for microarray analysis using Affymetrix Human Genome U133A. Results were analyzed using Genespring and Microarray Suite software.

Results

In response to treatment with a translational start site (TSS)-targeting ODN (SEQ ID NO:10), TS gene transcription was increased by 70% as compared to treatment with a control ODN (SEQ ID NO:9) (Figure 1). The increased TS gene transcription observed in cells treated with SEQ ID NO:10 did not change steady-state TS mRNA level (measured by RT-PCR), but resulted in a 35% increase in TS protein activity (Figures 2A, 2B and 3). However, the treatment with SEQ ID NO:10 did not alter the proliferation rate, cell cycle distribution or resistance to TS-targeting drugs (5-FUdR or raltitrexed) of HeLa cells (Figure 4A and B). The SEQ ID NO:10 ODN-mediated increase in TS gene transcription and protein activity, in the absence of TS mRNA change, was explored by microarray analysis to assess changes in the expression of genes other than TS. The expression of a small group of genes (54 genes) was up- or

downregulated twofold or more following treatment of HeLa cells with SEQ ID NO:10 and included genes encoding proteins involved in cell growth and apoptosis (Table 3). Genes that were significantly downregulated after treatment with SEQ ID NO:10 include: coatomer protein complex subunit alpha (alpha-COP), cofactor required for Sp1 transcriptional activation subunit 9 (CRSP), activating transcription factor 5 (ATF5), and retinoblastoma susceptibility (RB). Genes that were significantly upregulated after treatment with SEQ ID NO:10 include: adenylate kinase 2 (AK2), piccolo, AXL receptor tyrosine kinase, peroxisome assembly factor 2 (PAF-2), GAP associated tyrosine phosphoprotein, p62 (Sam68), pregnancy-specific beta-1 glycoprotein (PSG95), Fanconi anemia complementation group A (FANCA), and Fc of IgE high affinity receptor for alpha polypeptide.

Table 3: Gene expression changes in HeLa cells following treatment with TS antisense ODN SEQ ID NO:10

Decreased	Gene	Function
0.33, 0.46	Coatmer protein complex subunit alpha (alpha-COP)	Intracellular protein transport
0.46, 0.33	Cofactor required for Sp1 trancriptional activation subunit 9 (CRSP)	Mediates activator-dependent transcription
0.42, 0.06	Activating transcription factor 5 (ATF5)	Regulatory role in the differentiation of neuroprogenitor cells
0.44, 0.07	Retinoblastoma susceptibility (Rb)	Tumor suppressor
Increased	**Gene**	**Function**
1.9, 0.9	Adenylate kinase 2 (AK2)	Maintenance of ADP/ATP levels, translocated in apoptotic cells alongside cytochrome c
2.1, 0.9	Piccolo	Component of presynaptic cytomatrix
1.9, 1.0	Axl receptor tyrosine kinase	Mitogenic factor, role in apoptosis, cell adhesion, chemotaxis
1.9, 1.1	Peroxisome assembly factor-2 (PAF-2)	Putative ATPase
2.5, 0.7	GAP-associated tyrosine phosphoprotein p62 (Sam68)	Stimulates G1/S transition
4.4, 2.1	Pregnancy-specific beta-1 glycoprotein (PSG95)	Enhances growth and maturation of embryos
2.9, 2.1	Fanconi anemia complementation group A (FANCA)	Oxidative stress, redox dependent cell signalling
2.0, 2.0	Fc of IgE high affinity receptor for alpha-polypeptide	Production of cytokines

EXAMPLE 2: Analysis of Gene Expression Changes following TS Downregulation by Antisense Oligonucleotides.

Oligodeoxynucleotides

A fully phosphorothioated ODN with 2'-methoxy-ethoxy modification on the 6 nucleotides at both the 5'- and 3'-ends was used in the following Example: ODN SEQ ID NO: 2 (5'-GCCAGTGGCAACATCCTTAA-3'). The scrambled control ODN SEQ ID NO: 8 (5'-ATGCGCCAACGGTTCCTAAA-3') has the same base composition as ODN SEQ ID NO: 2, in random order, and is not complementary to any region of human TS. There are no other known human mRNAs with more than 15 bases of complementarity to any of the ODNs used.

ODN treatment and RNA isolation:

HeLa cells were plated at 1 x 10^6 cells per 75-cm^2 flask in 5 ml of medium. On the following day, ODNs (600 nM) were mixed with 6 μg/ml LipofectAmine 2000™ in serum-free medium for 15 min at room temperature to yield a 6 X transfection mix. The ODN:lipid mixture (1 ml) was added to each 75-cm^2 flask to yield final concentrations of 100 nM ODN and 1 ug/ml lipid. After 8 and 24 hours, RNA was isolated using TRIzol™ (Invitrogen), and quantified using a spectrophotometer.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR):

cDNA was prepared from 1 μg total RNA using MMLV reverse transcriptase. 2 % of the cDNA was used in PCR with glyceraldehyde-3-7,8 phosphate dehydrogenase (GAPDH) primers or TS primers. Products were resolved on 1.5% agarose gels and stained with ethidium bromide. Real-time PCR was performed on an ABI 7000 system.

Western Blotting:

Cell lysates (10 μg total protein) were resolved on SDS-PAGE (12% acrylamide), transferred to Hybond™ ECL membranes (Amersham) and blocked overnight in 5% skim milk powder in TBS-Tween™ at 4°C. Membranes were incubated for 4h with rabbit anti-TS PAb (0.125 μg/ml), washed and incubated with HRP-labelled anti-rabbit antibody. ECL Plus (Amersham) was used to visualize proteins.

Microarray hybridization and analysis:

RNA samples (10 μg) were sent to the London Regional Genomics Centre for processing and hybridization to Affymetrix™ human U133A arrays. Two control ODN SEQ ID NO:8-treated samples were pooled, while two TS antisense ODN SEQ ID NO:2-treated samples were

processed and analyzed independently. Microarray Suite 5.0 and Genespring™ 6.0 were used to analyze the data.

Results

HeLa cells were treated *in vitro* with TS antisense ODN SEQ ID NO: 2 or control scrambled ODN SEQ ID NO: 8, for 8 or 24 hours, and RNA extracted. RT-PCR confirmed down-regulation of TS mRNA relative to GAPDH mRNA levels following treatment with TS antisense ODN SEQ ID NO: 2 but not the controlled scrambled ODN SEQ ID NO: 8 at both times (Fig. 5A). TS mRNA levels relative to 18S rRNA levels, quantitated by real-time PCR, were reduced at both times by 95 % in cells treated with TS antisense ODN SEQ ID NO: 2 compared with control scrambled ODN SEQ ID NO: 8 (Fig. 5B). TS protein levels, as measured by Western Blot analysis (Fig. 5C), were decreased with TS antisense ODN SEQ ID NO: 2 treatment, relative to ODN SEQ ID NO: 8 or untreated controls.

Microarray analysis was performed as described above to detect changes in gene expression in HeLa cells treated *in vitro* with TS antisense ODN SEQ ID NO: 2 or control scrambled ODN SEQ ID NO: 8, for 8 or 24 hours. Scatter graphs show transcripts with altered expression in HeLa cells treated for 8 hours (Fig. 6A) or 24 hours (Fig. 6B) with TS antisense ODN SEQ ID NO: 2 (y-axis) compared with control scrambled ODN SEQ ID NO:8 (x-axis). Diagonal lines indicate 2-fold and 5-fold expression changes. The graph shows only those transcripts on the U133A array that fulfil the following criteria: present in all samples; increased (or decreased) in 2 of 2 ODN SEQ ID NO: 2-treated samples relative to ODN SEQ ID NO: 8-treated samples; statistically significant change ($p<0.005$) in both; signal log ratio > 0.5 or < -0.5 (Fig. 6A), or signal log ratio > 1.0 or < -1.0 (Fig. 6B).

Bioinformatics analysis of the U133A array data was performed using Genespring™, to categorize the changes in gene expression profiles. Analysis was performed on transcripts that were present in all samples and whose expression was altered > 2-fold in 2 of 2 ODN SEQ ID NO: 2-treated samples compared with ODN SEQ ID NO: 8-treated samples. The transcripts with decreased expression at 8 hours, but unchanged at 24 hours in response to treatment with ODN SEQ ID NO: 2, relative to treatment with ODN SEQ ID NO: 8, are detailed in Table 4. For example, genes downregulated at 8 hours include subunits of the anaphase promoting complex and origin recognition complex.

Microarray Suite 5.0 was also used to analyze the array data. The transcripts that were present in all samples and had signal log ratio < -1.0 in both 8 hour and both 24 hour TS antisense ODN SEQ ID NO:2-treated samples, or signal log ratio > 0.5 in all four samples, compared with control scrambled ODN SEQ ID NO: 8-treated samples were analyzed. The transcripts with altered expression are detailed in Table 5. For example, TS transcripts were decreased (signal log ratios ranged from -3.4 to -3.6). In addition, the expression of dihydropyrimidine dehydrogenase, the rate-limiting enzyme for catabolism of 5-FU, was also reduced at 8 and 24 hours (-0.5 and -1.0, respectively).

The transcripts that were significantly increased (> 2-fold) after 24 hours (but not 8 hours) in response to treatment with ODN SEQ ID NO: 2, relative to treatment with ODN SEQ ID NO: 8, are detailed in Table 6.

Table 4: Transcripts decreased after 8 hours but not 24 hours of ODN SEQ ID NO: 2 Treatment.

Affymetrix probe	Accession (NCBI)	Description
202672_s_at	NM_001674.1	activating transcription factor 3
211063_s_at	BC006403.1	NCK adaptor protein 1
208944_at	D50683.1	transforming growth factor, beta receptor II
209682_at	U26710.1	Cas-Br-M (murine) ectropic retroviral transforming sequence b
203735_x_at	N35896	PTPRF interacting protein, binding protein 1 (liprin beta 1)
212381_at	BF444943	ubiquitin specific protease 24
221419_s_at	NM_013307.1	non-functional folate binding protein
201883_s_at	D29805.1	beta-1,4-galactosyltransferase
216609_at	AF065241.1	thioredoxin delta 3 (TXN delta 3)
200630_x_at	AV702810	SET translocation (myeloid leukemia-associated)
214913_at	AB002364.1	disintegrin-like and metalloprotease (reprolysin type)
203386_at	AI650848	KIAA0603 gene product
212209_at	AL133033.1	KIAA1025 protein
203250_at	NM_014892.1	KIAA1116 protein
214001_x_at	AW302047	ribosomal protein S10
216246_at	AF113008.1	ribosomal protein S20
213642_at	BE312027	ribosomal protein L27
212044_s_at	BE737027	ribosomal protein L27a
202028_s_at	BC000603.1	ribosomal protein L38
221943_x_at	AW303136	ribosomal protein L38
AFFX-HUMRGE/M10098_5_at		M10098 Human 18S rRNA gene
AFFX-HUMRGE/M10098_3_at		M10098 Human 18S rRNA gene
AFFX-r2-Hs18SrRNA-5_at		M10098 Human 18S rRNA sequence, target bases 1-646
AFFX-r2-Hs18SrRNA-M_x_at		M10098 Human 18S rRNA sequence, target bases 647-1292
AFFX-r2-Hs18SrRNA-3_s_at		M10098 Human 18S rRNA sequence, target bases 1293-1938
AFFX-M27830_5_at		M27830 Human 28S ribosomal RNA gene
AFFX-r2-Hs28SrRNA-3_at		M11167 Human 28S rRNA sequence, target bases 1666-3330

Table 5: Transcripts with altered expression in HeLa cells treated with TS antisense ODN SEQ ID NO: 2.

Decreased expression in ODN SEQ ID NO: 2 vs ODN SEQ ID NO: 8 treated cells:

Treatment time:	8 hours		24 hours				
Replicate:	1	2	1	2			
Affymetrix probe	Signal log ratio				Accession (NCBI)	Unigene	Description
202589_at	-3.5	-3.4	-3.4	-3.6	NM_001071.1	Hs.82962	thymidylate synthase
213007_at	-1.5	-1.4	-2.1	-2	W74442	Hs.80961	polymerase (DNA directed), gamma
213008_at	-1.4	-1.5	-2.5	-2.3	BG403615	Hs.80961	polymerase (DNA directed), gamma
204957_at	-1.8	-1.8	-2.3	-2	NM_002553.1	Hs.153138	origin recognition complex, subunit 5
211212_s_at	-2.1	-1.7	-2	-2	AF081459.1	Hs.153138	origin recognition complex, subunit 5
209153_s_at	-1.2	-1	-1.7	-1.5	M31523.1	Hs.101047	transcription factor 3 (E2A immunogl
222182_s_at	-1.3	-1.5	-1.5	-1.4	BG105204	Hs.239720	CCR4-NOT transcription complex, su
203011_at	-1.9	-2.1	-1.7	-2.1	NM_005536.2	Hs.171776	inositol(myo)-1(or 4)-monophosphata
203367_at	-1.1	-1.2	-1.1	-1.1	NM_007026.1	Hs.91448	MKP-1 like protein tyrosine phosphat
222077_s_at	-2.8	-2.8	-3.2	-3.1	AU153848	Hs.23900	GTPase activating protein
210139_s_at	-1.5	-1.2	-1.9	-1.9	L03203.1	Hs.103724	peripheral myelin protein 22
217822_at	-2.1	-1.7	-2.5	-2.9	NM_016312.1	Hs.16420	Npw38-binding protein NpwBP
205807_s_at	-1	-1.2	-1.3	-1.3	NM_020127.1	Hs.283009	tuftelin 1
218911_at	-2.5	-2.6	-2	-1.8	NM_006530.1	Hs.4029	glioma-amplified sequence-41
202102_s_at	-1	-1	-1.5	-1.5	BF718610	Hs.278675	bromodomain-containing 4
212912_at	-1.4	-1.1	-1.3	-1	AI992251	Hs.184581	ESTs
218330_s_at	-1.5	-1.3	-1.1	-1.1	NM_018162.1	Hs.23467	hypothetical protein FLJ10633
212673_at	-1.6	-1.6	-1.6	-1.8	D42084.1	Hs.82007	KIAA0094 protein
211996_s_at	-1	-1.5	-1.8	-1.6	BG256504	Hs.110613	KIAA0220 protein
203386_at	-1.1	-1.2	-1	-1.4	AI650848	Hs.173802	KIAA0603 gene product
213154_s_at	-1.1	-1	-1.3	-1.4	AI934125	Hs.17411	KIAA0699 protein
204066_s_at	-1.1	-1.2	-1.6	-1.6	NM_014914.1	Hs.159377	KIAA1099 protein

Increased expression in ODN SEQ ID NO: 2 vs ODN SEQ ID NO: 8 treated cells:

Treatment time:	8 hours		24 hours				
Replicate:	1	2	1	2			
Affymetrix probe	Signal log ratio				Accession (NCBI)	Unigene	Description
218379_at	1.2	0.7	1.3	1.1	NM_016090.1	Hs.5887	RNA binding motif protein 7
202162_s_at	0.8	1	1.1	1	AI769416	Hs.26703	CCR4-NOT transcription complex, su
204234_s_at	1.3	0.6	1.2	1.6	AI476267	Hs.104382	zinc finger protein 195
209709_s_at	1	0.6	0.8	1.2	U29343.1	Hs.72550	hyaluronan-mediated motility recepto
200742_s_at	0.6	0.6	1.4	1.1	BG231932	Hs.20478	ceroid-lipofuscinosis, neuronal 2, late
214349_at	0.7	0.8	1.2	1.1	AV764378	Hs.163863	ESTs, similar to POL2_HUMAN RE1
202272_s_at	0.7	0.6	0.9	0.7	NM_015176.1	Hs.64691	KIAA0483 protein

Table 6: Transcripts increased after 24 hours (but not 8 hours) in HeLa cells treated with TS antisense ODN SEQ ID NO: 2.

Affymetrix probe	Accession (NCBI)	Unigene	Description
203889_at	NM_003020.1	2265	secretory granule, neuroendocrine protein 1 (7B2 protein) (SGN
218032_at	AF070673.1	76691	stannin
207205_at	NM_001817.1	12	carcinoembryonic antigen-related cell adhesion molecule 4 (CE
221128_at	NM_023038.1	278679	disintegrin and metalloproteinase domain 19 (meltrin beta) (AD
202379_s_at	AI361805	241493	natural killer-tumor recognition sequence
217976_s_at	NM_016141.1	266483	dynein light chain-A
219645_at	NM_001231.1	60708	calsequestrin 1 (fast-twitch, skeletal muscle)
211207_s_at	AF129166.1	14945	long-chain acyl-CoA synthetase 5
220460_at	NM_017435.1	279005	solute carrier family 21 (organic anion transporter), member 14
210941_at	AB006756.1	34073	BH (brain-heart) protocadherin (PCDH7)
203972_s_at	AB035307.1	7277	peroxisomal biogenesis factor 3
221923_s_at	AA191576	9614	nucleophosmin (nucleolar phosphoprotein B23, numatrin)
204234_s_at	AI476267	104382	zinc finger protein 195
204532_x_at	NM_021027.1	2056	UDP glycosyltransferase 1 family, polypeptide A9 (UGT1A9)
205626_s_at	NM_004929.2	65425	calbindin 1, (28kD) (CALB1)
208965_s_at	BG256677	155530	interferon, gamma-inducible protein 16
210969_at	AF116089.1	69171	protein kinase C-like 2
204404_at	NM_001046.1	110736	solute carrier family 12 (sodium potassium chloride transporters
206332_s_at	NM_005531.1	155530	interferon, gamma-inducible protein 16
212172_at	AW277253	171811	adenylate kinase 2
203134_at	NM_007166.1	7885	phosphatidylinositol binding clathrin assembly protein
201485_s_at	BC004892.1	79088	reticulocalbin 2, EF-hand calcium binding domain
221249_s_at	NM_030802.1		CEBP-induced protein
210095_s_at	M31159.1	77326	insulin-like growth factor binding protein 3
211097_s_at	BC003111.1	93728	pre-B-cell leukemia transcription factor 2
212417_at	BF058944	31218	secretory carrier membrane protein 1
213623_at	NM_007054.1	43670	kinesin family member 3A
206587_at	NM_006584.1	73072	chaperonin containing TCP1, subunit 6B (zeta 2)
207379_at	NM_005711.1	129764	EGF-like repeats and discoidin I-like domains 3
218006_s_at	NM_006963.1	108642	zinc finger protein 22 (KOX 15)
211711_s_at	BC005821.1		phosphatase and tensin homolog (mutated in multiple advance
207995_s_at	NM_014257.1	23759	CD209 antigen-like
221139_s_at	NM_015989.1	279815	cysteine sulfinic acid decarboxylase-related protein 2
208883_at	BF515424	278428	progestin induced protein
208486_at	NM_000798.1	143526	dopamine receptor D5
205543_at	NM_014278.1	71992	heat shock protein (hsp110 family)
205260_s_at	NM_001107.1	18573	acylphosphatase 1, erythrocyte (common) type
203824_at	NM_004616.1	84072	transmembrane 4 superfamily member 3 (TM4SF3)

The disclosure of all patents, publications, including published patent applications, and database entries referenced in this specification are specifically incorporated by reference in their entirety to the same extent as if each such individual patent, publication, and database entry were specifically and individually indicated to be incorporated by reference.

The embodiments of the invention being thus described, it will be obvious that the same may be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the invention, and all such modifications as would be obvious to one skilled in the art are intended to be included within the scope of the following claims.